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CHOLERA TOXIN B SUBUNIT AS A CARRIER PROTEIN TO STIMULATE A MUCOSAL IMMUNE RESPONSE[1]

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Horseradish peroxidase (HRP) was covalently coupled to the binding subunit of cholera toxin (CTB) via a two-step glutaraldehyde procedure. The HRP-CTB conjugate was characterized by physiochemical as well as immunochemical methods. Mice were immunized intraduodenally with the HRP-CTB conjugate, with HRP alone, or with a mixture of uncoupled CTB and HRP. The functionally active dose of CTB was 50 μg and the HRP dose was in the 30- to 90-μg range. Both IgA and IgG antibody responses were measured in serum, intestinal washes, and bile by using a solid phase immunoradiometric assay. Mice immunized with the HRP-CTB conjugate showed a significantly higher level of IgA anti-HRP in intestinal washes and bile, as well as increased levels of serum IgG anti-HRP. Animals that received only HRP or the mixture of CTB and HRP had reduced levels of HRP-specific antibody of either class in both gut washes and bile. The IgA anti-HRP responses in the gut washes were 33- to 120-fold higher when the conjugate was used as the immunogen in comparison with immunization with the CTB + HRP or the HRP alone. Vaccines to stimulate mucosal immunity to any antigenic determinant might thus be prepared by covalent conjugation to effective mucosal immunogens such as CTB.

Interest in covalent protein-protein conjugates has grown rapidly since enzymes were first coupled to antigens and antibodies in the search for effective markers in histochemical studies (1–3). The use of other types of protein conjugates in immunology is not a new concept. It has long been known that an immune response to a small, otherwise nonimmunogenic molecule can be elicited if that molecule is coupled to a larger, immunogenic protein. More recently, interest has turned to coupling polysaccharides or chemically synthesized peptides to protein carriers to stimulate immunity to various pathogenic bacteria and viruses. Chu and co-workers (4) looked at parenteral immunity in mice after immunization with conjugates of bacterial capsular polysaccharides and tetanus toxoid. Muller *et al.* (5) and Green and co-workers (6) studied the anti-influenza response after parenteral immunization of rabbits with conjugates of either hemocyanin or tetanus toxoid, and they chemically synthesized peptides from the hemagglutinin molecule of influenza virus. Lerner *et al.* looked at a similar system in rabbits (7) and in chimpanzees (8) in which they used a conjugate of hemocyanin and chemically synthesized peptides from the surface antigen of hepatitis B virus.

Because exposure to many pathogens is initially via a mucosal surface, the stimulation of a mucosal immune response in addition to a systemic response would likely be beneficial. It was noted by several investigators, however, that most nonliving, nonreplicating antigens are relatively ineffective at eliciting a mucosal (IgA) response (9, 10). In fact, antigens may induce systemic tolerance when they are administered orally (11, 12). An interesting exception to these generalizations is the toxin produced by *Vibrio cholerae*, which has been shown to be a potent mucosal immunogen by a number of investigators (13–18). We chose the binding subunit of cholera toxin (CTB)[3] as a model carrier protein for mucosal immunization, because it also stimulates mucosal immune responses but is not itself toxic. Cholera toxin and its B subunit are known to bind to the G_{M1} ganglioside found in cell membranes. It has been postulated that this ability confers upon them their mucosal immunogenicity, by aiding uptake by M cells or by trapping mucosal lymphocytes or macrophages or both (15). These experiments were designed to determine whether CTB could stimulate mucosal immunity to an attached protein that is not normally immunogenic at mucosal surfaces. Horseradish peroxidase (HRP) was used as the experimental immunogen of interest because the coupling procedure with glutaraldehyde is well defined for this protein (19). The results suggest that CTB is indeed an effective carrier for stimulating mucosal immunity to a second, covalently linked protein.

MATERIALS AND METHODS

Mice. Female BALB/c BYJ mice (Jackson Laboratory, Bar Harbor, ME), 12 to 16 wk of age, were used for all experiments.

Reagents. Horseradish peroxidase (HRP), cholera toxin B subunit (CTB), glutaraldehyde, bovine brain gangliosides (type II), soybean trypsin inhibitor, phenylmethylsulfonyl fluoride (PMSF), and bovine serum albumin (BSA) were all purchased from Sigma Chemical Co., St. Louis, MO. Affinity-purified goat anti-mouse IgG and affinity-purified goat anti-mouse IgA were obtained from Kirkegaard & Perry Laboratories, Gaithersburg, MD; goat anti-rabbit IgG was acquired from Miles Laboratories, Elkhart, IN; and goat anti-mouse IgG and goat anti-mouse IgA were purchased from Cappel Laboratories, West Chester, PA.

Affinity purification of antisera. Goat antisera to rabbit IgG was affinity purified on glutaraldehyde cross-linked rabbit IgG by the

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[3] Abbreviations used in this paper: CTB, cholera toxin B subunit; HRP, horseradish peroxidase; PMSF, phenylmethylsulfonyl fluoride; TBS, Tris-buffered saline.

method of Avrameas and Ternynck (20).

Conjugation of HRP to CTB. HRP was covalently coupled to CTB via a two-step glutaraldehyde procedure (19). Approximately 20 mg of HRP were dissolved in 200 μl of 0.1 M phosphate buffer, pH 6.8, and 10 μl of 25% glutaraldehyde were added. The mixture was incubated overnight (18 hr) in the absence of light at room temperature, and was then applied to a blue dextran calibrated Biogel P-100, fine, gel filtration column (1.5 x 50 cm). Fractions from the third peak, containing monomeric activated HRP, were pooled and mixed with 2 mg of CTB in 1 ml PBS (0.01 M sodium phosphate, 0.1 M NaCl, pH 7.3), and 200 μl of 1 M carbonate buffer, pH 9.5. This mixture was incubated for 24 hr at 4°C and was then applied to a blue dextran calibrated Sephadex G-100, superfine, column (1.5 x 50 cm). The first two peaks, which contained the HRP-CTB conjugate, were pooled and characterized further. The concentration of each protein in the conjugate preparation was estimated in the following manner. The ratio of the absorbence at 403 nm to that at 280 nm for the activated HRP pool used for conjugation was determined to be 2.70. This value was used to calculate the relative contributions of each protein to the absorbence at 280 nm in the final elution profile of the conjugate by using the equations: a) A_{280} due to HRP = $A_{403}/2.70$; b) Concentration of HRP (mg/ml) = $A_{403} \times 0.4$ (21); c) A_{280} due to CTB = A_{280} total − A_{280} due to HRP; d) Concentration of CTB (mg/ml) = (A_{280} due to CTB)/0.956 (22).

Antisera. Rabbit antisera to CTB was obtained 4 wk after injection of a New Zealand White rabbit (Small Stock, Pea Ridge, AR) with 400 μg of CTB emulsified in complete Freund's adjuvant (CFA). Rabbit antisera to HRP was obtained by the injection of 5 mg of HRP in CFA twice, 1 wk apart, followed by injection of 12 mg HRP in CFA 5 wk later. Serum was obtained 1 wk after the final injection.

Iodination of proteins. All proteins were iodinated by the chloramine-T method of Sonoda and Schlamowitz (23).

Monoclonal antibodies. Mouse monoclonal antibodies of the IgG and IgA classes specific for CTB were the gift of Drs. John Cebra and Juliet Fuhrman. The concentration of mouse antibody in the hybridoma culture supernatants was determined by the method of radial immunodiffusion described by Mancini *et al* (24).

Red blood cell binding assay. Fresh rat red blood cells (Fisher rats) were diluted 1/100 (v/v) in 10 mg/ml BSA in PBS and 250 μl of this suspension were added to microcentrifuge tubes precoated with 10 mg/ml BSA. A standard curve of CTB binding to the red blood cells was generated by the addition of 100 μl of the appropriate dilution of CTB in 10 mg/ml BSA in the presence or absence of exogenous gangliosides (10 μg/ml in 10 mg/ml BSA). This mixture was incubated for 30 min at room temperature. The cells were sedimented by centrifugation at 2000 × G for 10 min and were washed three times in 10 mg/ml BSA. The cells were then incubated for 30 min with 200 μl of a 1/10 dilution of rabbit anti-CTB in 10 mg/ml BSA. The cells were again sedimented and washed and 200 μl of ^{125}I-goat anti-rabbit IgG were added and incubated for 1 hr at room temperature. After a final centrifugation and wash, the amount of binding was determined by counting the cells in a gamma counter. The amount of CTB in the conjugate still capable of binding to the red blood cells was determined by extrapolation of the amount bound of a dilution of the conjugate from the standard curve obtained.

Electrophoresis. Immunoelectrophoresis was performed by the method of Ouchterlony and Nilsson (25). The conjugate was analyzed further by SDS-PAGE in a 7% acrylamide gel, in the presence of 8 M urea (26), with a mini-gel apparatus (Idea Scientific, Corvallis, OR) (27). The protein bands were visualized by a silver staining method (28).

Immunoblot of SDS-urea gel. Immunoblots were done by a modification of the methods of Bowen *et al.* (29) and Beisiegel *et al.* (30). Immediately after electrophoresis, protein bands on the SDS-urea gel were adsorbed onto nitrocellulose paper (Schleicher & Schuell, Keene, NH), which had been premoistened with TBS (Tris-buffered saline, 0.05 M Tris, 0.1 M NaCl, 0.02% NaN_3, pH 7.6). Adsorption occurred by passive diffusion for 2 hr at room temperature, with a 700-g weight used to apply pressure and to aid the transfer of the protein. The nitrocellulose paper was then placed in a small petri dish containing 50 ml of TBS and 50 mg/ml BSA overnight at 4°C. The blot was incubated with 50 ml of either a 1/500 dilution of rabbit anti-HRP serum in 50 mg/ml BSA or a 1/100 dilution of rabbit anti-CTB for 2 hr at room temperature. The paper was rinsed twice with TBS, twice with TBS + 0.5% Tween 20, and twice again with TBS. It was then incubated with 15 μg of ^{125}I-goat anti-rabbit IgG (specific activity approximately 0.5 μCi/μg) in 50 ml of TBS + 50 mg/ml BSA, for 1 hr at room temperature. The blot was rinsed twice in the Tween washing solution and then left overnight in the Tween solution. After the paper was air-dried, the protein bands were visualized by autoradiography. In the case of the anti-CTB blot, the autoradiograph was incubated for 48 hr, and in the case of the anti-HRP blot, the bands were visualized after a 30-hr incubation—both at −70°C with an enhancing screen.

Immunization of mice. Mice were fasted overnight, anesthetized with methoxyflurane (Metofane, Pitman-Moore, Washington Crossing, NJ), and then immunized intraduodenally through a laparotomy. On day 0, each animal received 100 μl of conjugate containing 50 μg of active CTB and 30 to 90 μg of coupled, activated HRP depending on the conjugate preparation used. Control animals received either a comparable dose of activated HRP alone, or a mixture of CTB + activated HRP (50 μg of CTB and 30 to 90 μg activated HRP). The immunization was repeated on day 14, and the animals were sacrificed on day 19. At this time, blood, bile, and gut washings were obtained. To minimize loss of antibody or antigen in the assay through degradation by endogenous proteases in the gut wash, the gut washes were treated with enzyme inhibitors (31). The method used was one developed by Elson *et al.* (32). The entire small intestine was removed and rinsed with 1 ml of PBS. These washes were immediately centrifuged at 4800 × G for 10 min. Solid matter was discarded, and 100 μl of a 1 mg/ml soybean trypsin inhibitor, 50 mM EDTA solution, and 10 μl of 100 mM PMSF in 95% ethanol were added. The washes were centrifuged again at 27,000 × G for 20 min. Supernatants were removed to fresh storage tubes, with the addition of 10 μl of 5% sodium azide and 10 μl of 100 mM PMSF. The samples were allowed to stand at room temperature for 15 min, at which time 50 μl of normal goat serum were added. The final concentrations of the added reagents in the gut wash were 1.7 mM PMSF, 1.6% ethanol, 85 μg/ml soybean trypsin inhibitor, 4 mM EDTA, and 0.04% sodium azide. The gut washes were then stored at −20°C until assayed for antibody activity. Normal gut wash, to be used as a control, was treated in exactly the same manner. All samples of serum, gut washes, and bile were diluted in 50% normal goat serum for subsequent assay.

Two groups of mice were also immunized i.p. with the conjugate preparation or with free HRP. These animals received 100 μl of conjugate that contained 10 μg of active CTB and 5.8 μg coupled, activated HRP, or 10 μg of activated HRP alone. They were immunized on days 0 and 14, and serum was obtained on day 19.

Immunoradiometric assay. Immulon 2 remove-a-well strips (Dynatech Laboratories, Inc., Alexandria, VA) were passively coated with 50 μl of 10 μg/ml CTB or activated HRP in 0.1 M carbonate buffer, pH 9.5, overnight at 4°C. The wells were washed once with a Tween wash solution (0.05% Tween 20, 0.05 M phosphate, pH 7.3) and twice with PBS. The wells were post-coated with 100 μl of 10% normal goat serum in PBS for 1 hr at room temperature. After this incubation, each well was washed as above, and they were stored moist at 4°C until used. Just before use, each well was washed three times with the Tween washing solution. The assay was performed by placing 50 μl of the appropriately diluted sample of serum, gut wash, or bile into a well coated with the antigen of interest. After a 6-hr room temperature incubation in a humidity chamber and three washes with Tween, 50 μl of a radiolabeled, class-specific antibody, diluted in 10% normal goat serum, were added to each well. This mixture was allowed to stand overnight at room temperature in a humidity chamber, and the plate was washed again three times with Tween. The wells were broken apart and placed in 12 x 75 mm test tubes to be counted in a gamma counter. A standard curve was generated on CTB-coated wells by using mouse monoclonal antibodies specific for CTB of either the IgG or IgA class, diluted in the appropriate normal fluid. All samples were analyzed in duplicate. The amount of specific antibody in the experimental samples was extrapolated from the standard curves; thus, unimmunized animals were used as the zero reference value. Previous experiments had shown that a standard curve generated with the use of a polyclonal antiserum of known antibody concentration gave the same results as this system with monoclonal antibody used as the standard.

Statistics. The statistical significance of the antibody responses was determined by using Students' paired *t*-test.

RESULTS

Conjugate preparation and characterization. Activation of HRP with glutaraldehyde produced monomeric HRP and some higher polymers of HRP, as can be seen in Figure 1. Only monomeric activated HRP obtained from the third peak was used to conjugate t CTB, however, to help simplify the characterization of the final product. Figure 2 shows the column profile obtained after gel filtration of the final conjugate preparation. The ratio of the absorbence of activated HRP at 403 nm to the absorbence at 280 nm before conjugation can be used to estimate how much of the absorbence at 280 nm is due

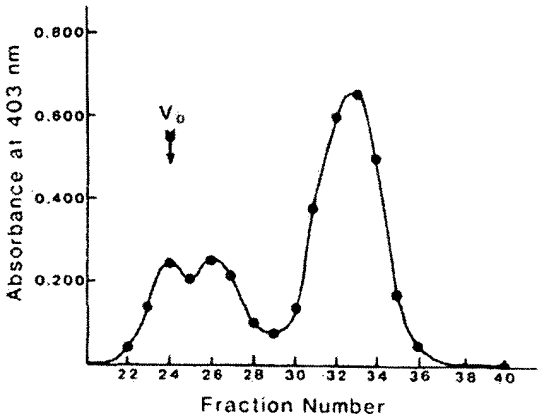

Figure 1. Gel filtration of glutaraldehyde-activated HRP. A 1.5 x 50 cm column was prepared by using Biogel P-100, fine (BioRad) equilibrated in physiologic saline. Total column volume was 80 ml; sample volume was 210 μl; fraction volumes were 1 ml. Flow rate was 8 ml/hr. Void volume was determined by using blue dextran (indicated by arrow).

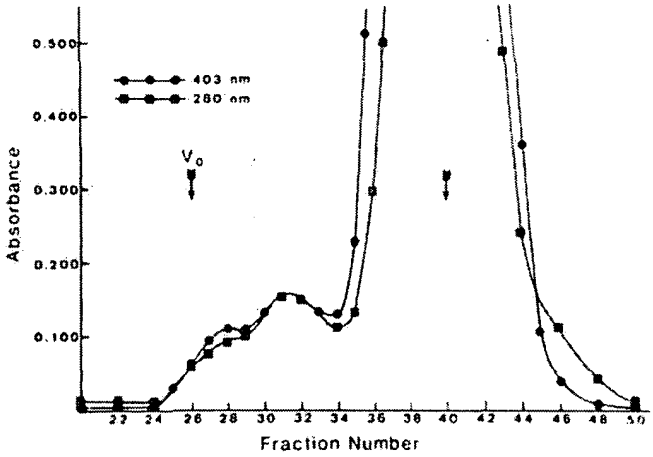

Figure 2. Gel filtration of the HRP-CTB conjugate preparation. A 1.5 x 50 cm column was prepared by using Sephadex G-100, superfine, equilibrated in physiologic saline. Total column volume was 80 ml; sample volume was approximately 4 ml; fraction volumes were 1 ml. Flow rate was 5.5 ml/hr. Void volume was determined by using blue dextran (indicated by first arrow). Second arrow, elution volume of free HRP and free CTB.

to each protein present in the conjugate. By using this method, it was determined that the first peak obtained after gel filtration corresponds to an HRP:CTB ratio of 2:1; the second peak is made up of conjugate in a 1:1 ratio of the two proteins. Both of these peaks were pooled and concentrated and used for further characterization and immunization. The yields observed were approximately 6 to 7% of the total HRP used, and 30 to 40% of the CTB.

Ouchterlony double diffusion in agarose gels was employed to analyze the conjugate, by using the rabbit antisera to HRP and CTB. The results are shown in Figure 3*A*. In each case, the lower well contained the antigen: conjugate (well c), conjugate plus free CTB (well d), conjugate plus free HRP (well e), or free CTB plus free HRP (well f). Well a contained a dilution of the rabbit anti-CTB serum, and well b contained a dilution of the rabbit anti-HRP serum. These combinations were used to determine whether the conjugate preparation contained either free HRP or free CTB. When either unconjugated HRP or CTB were added to the conjugate preparation, the Ouchterlony analysis indicated a reaction of partial identity, shown

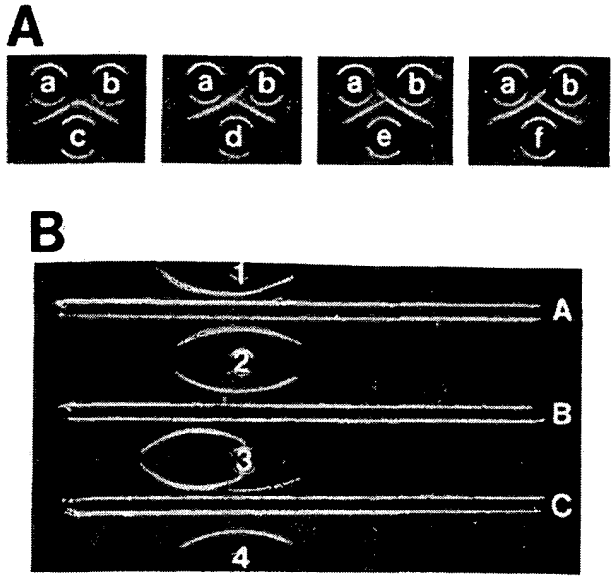

Figure 3. Ouchterlony double diffusion and immunoelectrophoresis of the HRP-CTB conjugate preparation. *A*, Ouchterlony analysis. Well a contained rabbit anti-CTB serum; well b contained rabbit anti-HRP serum. Lower wells contained: c: HRP-CTB conjugate; d: HRP-CTB plus free CTB; e: HRP-CTB plus free HRP; f: free HRP and free CTB. *B*, Immunoelectrophoresis. A mixture of free CTB and free HRP was electrophoresed in wells 1 and 3, the conjugate preparation in wells 2 and 4. Trough A contained rabbit anti-CTB; trough B contained rabbit anti-HRP; trough C contained a mixture of the two antisera.

by patterns d and e. When there was no conjugate present, the pattern observed was that of non-identity (pattern f). As can be seen in the pattern in which only conjugate was present (pattern a), the reaction is that of identity. Therefore, within the limits of detection of this system, there was apparently no free CTB or free HRP in the conjugate preparation.

Immunoelectrophoresis was also used to characterize the conjugate preparation further (Fig. 3*B*). The conjugate preparation was electrophoresed in wells 2 and 4, and a mixture of free HRP and free CTB was electrophoresed in wells 1 and 3. The first trough, A, contained rabbit anti-CTB, the second trough, B, contained rabbit anti-HRP, and the lower trough, C, contained a mixture of the two antisera. Under the conditions of the electrophoresis, CTB did not migrate from the origin, whereas HRP migrated slightly to the left of the origin, or toward the cathode. As shown in Figure 3*B*, the mixture of free CTB and free HRP did result in the formation of two distinct precipitin arcs when both antisera were used in the trough (well 3 and the lower trough, C), whereas the conjugate preparation showed only one precipitin arc in the presence of both antisera (well 4 and trough C). Again, this result indicates that there was no free HRP or CTB in the conjugate preparation.

SDS-urea polyacrylamide gels were also used to characterize the conjugate further. CTB is a pentamer of identical subunits that are held together by strong noncovalent interactions (33). In the presence of SDS and urea, most of these interactions, but not necessarily all, are disrupted and the protein migrates as one major band corresponding to a m.w. of roughly 11,000 (26). Figure 4*A* shows the results obtained after electrophoresis of the conjugate and the appropriate standards in the presence of SDS and urea. Lane 1 contains m.w. standards ranging from 29,000 to 205,000. Lane 2 contains intact

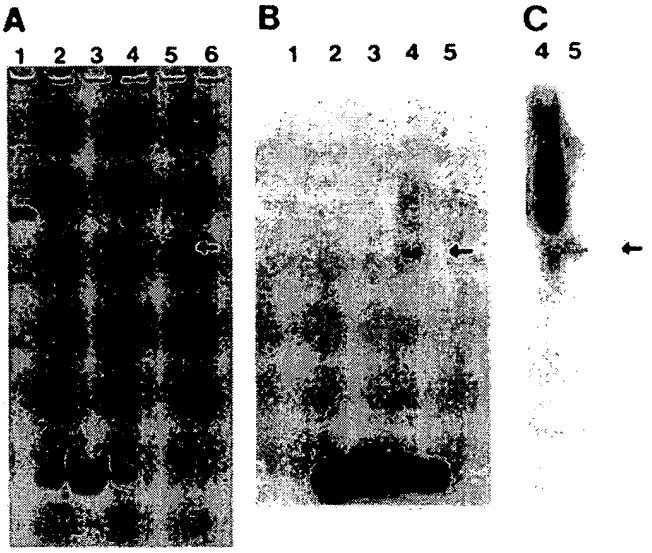

Figure 4. SDS/urea-PAGE and immunoblots of the conjugate preparation. *A*, a 7% acrylamide mini-slab gel was prepared containing 8 M urea. Samples also contained 8 M urea, the running buffer did not. The gel was electrophoresed for 1 hr at 100 V constant voltage. Protein bands were visualized by a silver staining method. Lane 1 contains the following m.w. standards: carbonic anhydrase, 29,000; ovalbumin, 45,000; BSA, 66,000; phosphorylase B, 97,400; β-galactosidase, 116,000; and myosin, 205,000. Lane 2 contains cholera toxin, lane 3, the CTB. Lane 4 contains conjugate preparation. Lanes 5 and 6 contain activated HRP from the third and second peaks, respectively, from the Biogel P-100 column (see Fig. 1). *B*, immunoblot of SDS/urea-PAGE gel with rabbit anti-CTB. Protein was blotted onto nitrocellulose paper by passive diffusion. Nitrocellulose paper was incubated first with rabbit anti-CTB, and then with ^{125}I-goat anti-rabbit IgG. Bands were visualized by autoradiography for 48 hr at −70°C with an enhancing screen. *C*, immunoblot with rabbit anti-HRP. This blot was prepared in the same manner as described above, except the first incubation after transfer onto the paper was with rabbit anti-HRP serum. Bands were visualized after a 30-hr incubation at −70°C with an enhancing screen.

cholera toxin, and shows the two bands corresponding with the A subunit and monomeric B subunits. Lane 3 contains only the B subunit of cholera toxin. It should be noted that under the conditions of the electrophoresis, the B subunit present in this lane is completely dissociated and migrates as the 11,000 m.w. monomers. Lane 4 contains the conjugate preparation, and lanes 5 and 6 contain activated HRP from the third and second peaks, respectively, from the Biogel P-100 column. As can be seen in lane 4, the conjugate preparation resulted in a number of bands, corresponding in m.w. to free monomeric CTB subunits, free HRP, and some fainter bands of higher m.w., which were postulated to consist of HRP coupled to one or more of the individual subunits from CTB. Because there is a band corresponding to the free B subunit monomers, it is possible that there was some pentameric CTB in the conjugate preparation that did not have any HRP attached to it. Because there was only one or two HRP molecules coupled per CTB molecule, however, it is likely that the HRP is coupled to only one, or at best two or three, of the monomers in any given pentameric molecule of CTB. Therefore the other monomers would be free upon dissociation by SDS and urea to migrate as the 11,000 dalton subunits.

Immunoblots were used to determine which of the other bands in the SDS gel contained CTB, which contained HRP, and which contained both proteins. The results from the anti-CTB immunoblot (Fig. 4*B*) suggest that the band present in the conjugate preparation that corresponds to a m.w. of approximately 50,000 (indicated by arrow) does contain some CTB subunits. Because this band migrates to the same extent as activated HRP (lane 5), which has a m.w. of only 40,000, plus an unknown amount of glutaraldehyde, it is probably not due to pentameric CTB, which has a m.w. of 55,000. Rather, it is postulated that it consists of four B subunit monomers that are still associated. All of the other bands present in the conjugate preparation also appear to contain CTB. The anti-HRP immunoblot (Fig. 4*C*) indicates that very little of the band corresponding to the m.w. of free HRP reacts with the anti-HRP antisera (arrow). This finding supports the results of the anti-CTB blot, which suggested that this band was due to remaining aggregates of four CTB subunits. All of the higher m.w. bands reacted with both the anti-HRP and the anti-CTB, which is what would be expected if the HRP was covalently coupled to one or more CTB subunits.

The conjugate preparation used to immunize the mice thus appears to consist of HRP_1-CTB and HRP_2-CTB. The results of the SDS-PAGE and immunoblots, which are complicated and somewhat difficult to interpret, suggest that the CTB and HRP present in the original conjugate mixture are indeed coupled to one another. These results, taken along with the results of the Ouchterlony analysis and immunoelectrophoresis, support the conclusion that there is no free pentameric CTB and very little, if any, free HRP in the mixture. In light of the antibody responses observed after immunization with the conjugate *vs* those seen for the mixture of the free proteins or HRP alone (see below), the presence of a small amount of free CTB or free HRP would not change the interpretation of the results.

Because the hypothesis concerning the effectiveness of CTB as a mucosal immunogen relies on the ability of CTB to bind to the G_{M1} ganglioside (15), a red blood cell binding assay was developed to determine if the binding ability of CTB was compromised during the conjugation procedure. Free CTB was used to generate a standard curve of the binding of CTB to rat red blood cells, which contain an endogenous G_{M1} ganglioside in their membrane. Exogenous gangliosides were added in some cases to assure that the binding of the CTB to the red blood cells was indeed specific. The standard curve generated is shown in Figure 5. As indicated, in the presence of exogenous gangliosides, essentially all binding of the CTB to the red blood cells was inhibited, which indicates that the binding of the CTB to the red blood cells was via the ganglioside. This assay should therefore be a good indicator of the remaining ability of the conjugate to bind to gangliosides. Dilutions of the conjugate preparations were assayed at the same time the standard curves were generated, maintaining the concentration of CTB in the conjugate within the range of the standard curve. Depending on the conjugate preparation tested, the binding ability of the CTB in the conjugate ranged from 35 to 95% of the total amount of CTB present, based on a total CTB concentration determined spectrophotometrically. It was discovered that repeated freezing and thawing of the conjugate resulted in a loss of its binding ability, as indicated by the 35% value. With later conjugate preparations, freezing and thawing was kept to a minimum, and this resulted in a much higher retention of the binding ability of the CTB in the conjugate.

Antibody responses after intraduodenal immuniza-

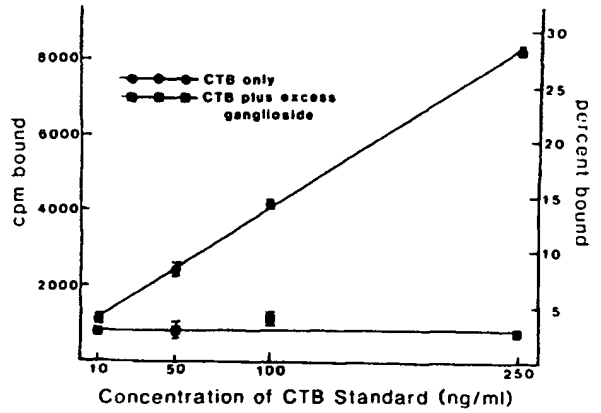

Figure 5. Standard curve of CTB binding to rat red blood cells. CTB in the presence (*closed squares*) or absence (*closed circles*) of exogenous gangliosides was incubated with rat red blood cells. After centrifugation and washing, cells were incubated with rabbit anti-CTB serum. Cells were again sedimented, washed, and incubated with ^{125}I-goat anti-rabbit IgG. After a final centrifugation and wash, cells were counted in a gamma counter to determine the amount of CTB bound. Amount of CTB in a conjugate preparation still capable of binding to the red blood cells was determined by incubating a dilution of the conjugate preparation in a similar manner, and extrapolating the amount of active CTB from the standard curve.

TABLE I

IgA anti-HRP responses in intraduodenally immunized mice[a]

Immunogen	IgA Anti-HRP (ng/ml)[b]		
	Gut wash	Bile	Serum
HRP alone	0.23 ⁑ 1.4 (10)	13 ⁑ 2.2 (8)	7.9 ⁑ 1.1 (10)
HRP + CTB mixture	0.38 ⁑ 1.8 (5)	22 ⁑ 1.3 (3)	8.4 ⁑ 1.1 (6)
HRP-CTB conjugate	10.1 ⁑ 1.3 (7)[c]	110 ⁑ 1.5 (7)[d]	10.0 ⁑ 1.1 (8)

[a] Mice were immunized intraduodenally on days 0 and 14 with 100 μl of conjugate containing 50 μg of active CTB and 30 to 90 μg coupled, activated HRP, or with comparable doses of activated HRP alone, or the mixture of activated HRP and CTB. Serum, gut wash, and bile were obtained on day 19. All samples were analyzed in duplicate by using a solid phase immunoradiometric assay. Unimmunized animals were used as the zero reference point.

[b] Geometric mean and standard error of the mean are shown. The number of animals in each group is in parentheses.

[c] $p < 0.005$ *vs* immunization with HRP alone or with the mixture.

[d] $p < 0.005$ *vs* HRP alone; $p < 0.025$ *vs* the mixture. Serum responses are not significantly different.

TABLE II

IgG anti-HRP responses in intraduodenally immunized mice[a]

Immunogen	IgG Anti-HRP (ng/ml)[b]		
	Gut wash	Bile	Serum
HRP alone	<1 (10)[c]	0.6 ⁑ 5.8 (5)	61 ⁑ 1.1 (10)
HRP + CTB mixture	<1 (5)	0.9 ⁑ 9.4 (3)	51 ⁑ 1.1 (6)
HRP-CTB	4.7 ⁑ 2.0 (7)[d]	5.6 ⁑ 5.2 (5)	582 ⁑ 1.2 (8)[d]

[a,b] See footnotes to Table I.

[c] All responses were equal to or less than the normal mouse controls.

[d] $p < 0.005$ *vs* HRP alone or *vs* the mixture. Bile responses are not significantly different.

tion. Antibody responses to HRP after intraduodenal immunization with conjugate, free HRP, or the mixture of free HRP and free CTB are shown in Tables I and II. All values were extrapolated from standard curves generated on CTB-coated wells by using mouse monoclonal antibodies specific for CTB of either the IgG or IgA class, diluted in the appropriate normal fluid. By using this method, unimmunized mice yield the zero reference value. Table I shows the IgA anti-HRP responses after intraduodenal immunization, with the results expressed as the geometric mean of the antibody responses. The results indicate that immunization with the HRP-CTB conjugate yields an IgA anti-HRP response in gut washes and bile that is significantly greater than the response to either HRP alone or the mixture of free CTB and free HRP ($p < 0.005$, except for the bile IgA anti-HRP, when conjugate *vs* mixture was significant at $p < 0.025$.) The IgA anti-HRP responses seen in the serum, however, were not significantly different. Table II shows the IgG anti-HRP responses after intraduodenal immunization. In the case of the IgG response to HRP, the antibody levels seen in the serum and gut wash when the conjugate was used as the immunogen were significantly higher than for the other immunogens ($p < 0.005$). The IgG anti-HRP responses seen in the bile were highly variable. In the HRP- and mixture-immunized groups, this can be attributed to a response by only one animal within each group. The conjugate-immunized mice, however, showed a consistent, albeit small, IgG response to HRP in the bile.

The anti-CTB responses in mice immunized with either the conjugate or with the mixture of CTB + HRP were also measured (results not shown). As expected, both groups responded well to the CTB as the immunogen, and the animals that received the mixture of the free proteins generally showed a higher anti-CTB response than did the conjugate immunized mice. This could be due to the loss of some antigenic sights on CTB after conjugation to HRP. The IgA anti-CTB responses in serum and gut wash ranged from 42 ⁑ 1.6 ng/ml to 172 ⁑ 1.4 ng/ml, and in the bile they were considerably higher: 4143 ⁑ 1.2 ng/ml for the mixture-immunized mice, and 1578 ⁑ 1.2 ng/ml for the conjugate-immunized mice. As seen in the anti-HRP response, the serum IgG anti-CTB response was orders of magnitude higher than the serum IgA anti-CTB response. The IgG anti-CTB responses were: 80 ⁑ 1.4 μg/ml for the animals that received the mixture, and 20 ⁑ 1.3 μg/ml for animals immunized with the conjugate. There was also some CTB-specific IgG in the gut washes and the bile of these animals, and these responses ranged from 26 ⁑ 1.6 ng/ml to 1054 ⁑ 1.1 ng/ml.

Antibody responses after i.p. immunization. Two additional groups of mice were immunized i.p. with the conjugate or with free HRP. These results are shown in Table III. Although there was no anti-HRP specific serum IgA present (all values were equal to or less than the normal mouse controls), there was a striking difference in the IgG response to HRP when HRP alone or HRP

TABLE III

Antibody responses to HRP and CTB after i.p. immunization[a]

	Anti-HRP Responses[b] (μg/ml)		Anti-CTB Responses[b] (μg/ml)	
	IgG	IgA	IgG	IgA
HRP alone	0.047 ⁑ 1.2	<0.001[c]	ND[d]	ND
HRP-CTB	25 ⁑ 1.2[e]	<0.001	144 ⁑ 1.2	0.016 ⁑ 1.1

[a] Mice were immunized on days 0 and 14 with a conjugate preparation containing 10 μg of active CTB and 5.8 μg coupled, activated HRP, or with 10 μg activated HRP alone. Serum was collected on day 19. Antibody levels were determined by using a solid phase immunoradiometric assay. All samples were analyzed in duplicate. Unimmunized animals were used as the zero reference point.

[b] Geometric mean and standard error of the mean are shown. Five animals in each group.

[c] All responses were equal to or less than the normal mouse controls.

[d] Not determined.

[e] $p < 0.005$ *vs* immunization with HRP alone.

conjugated to CTB was used as the immunogen ($p < 0.005$). Again the response to CTB in the conjugate-immunized mice was high, with the serum IgG values at 144 ± 1.2 μg/ml, and the serum IgA at 16 ± 1.1 ng/ml.

DISCUSSION

The results of this study indicate that CTB can function as an effective carrier to stimulate mucosal immunity to a protein that is not normally immunogenic at mucosal surfaces. When immunized intraduodenally with a covalent conjugate of HRP and CTB, mice respond to both proteins with the production of local IgA, as seen in the gut washes and bile, as well as by producing high levels of serum IgG. There was also antigen-specific IgG in the gut washes and bile of the HRP-CTB conjugate-immunized animals. The origin and significance of this antibody is not clear, but the presence of antigen-specific IgG in gut washes was reported by Pierce *et al.* (34) and by Holmgren *et al.* (35). Mice immunized intraduodenally with HRP alone did not show a significant response to HRP in any of the fluids tested, nor did the animals that received a mixture of CTB + HRP. Thus, the response to HRP in the conjugate-immunized mice appears to depend on the covalent nature of the association of HRP to CTB, and is therefore similar to the carrier effect seen for haptens. The enhanced immunogenicity to HRP was probably not due simply to the increase in molecular size when the conjugate was used as the immunogen, because hemocyanins (m.w. = 300,000 to 9,000,000) (36) are not effective mucosal immunogens (11, 18), even though they are potent systemic immunogens. However, further studies with aggregates of HRP or HRP coupled to a poor mucosal immunogen would help to define the observed phenomenon more clearly. The responses seen after i.p. immunization also indicate that CTB is an extremely effective carrier. The response to HRP when the conjugate was used as the immunogen was 500-fold greater than the response when HRP was the immunogen, even though the dose of HRP was lower in the conjugate-immunized mice. A similar carrier-type of effect was noted by Northrup and Fauci (37), who immunized mice with a mixture of cholera toxin and sheep red blood cells (SRBC). They reported an increased response to the SRBC when cholera toxin was present, and referred to the "adjuvanticity" of cholera toxin. In light of what is now known about the binding of cholera toxin to the G_{M1} ganglioside, it is possible that in their system, cholera toxin physically bound to the SRBC and acted more as a carrier than as an adjuvant. Further investigation into this phenomenon would be needed, however, to characterize fully the mechanism by which cholera toxin, and its B subunit, seem to enhance both the parenteral and mucosal immune response.

Other investigators have been working with protein conjugates in the interest of stimulating immunity to viral and bacterial antigens. Chu and co-workers (4) looked at parenteral immunity in mice after immunization with conjugates of bacterial capsular polysaccharides and tetanus toxoid. These researchers introduced the idea of "useful" and "nonsense" carriers; it would be beneficial to stimulate immunity to a useful carrier (tetanus toxoid) as opposed to stimulating immunity to an irrelevant carrier (hemocyanin) in the case of immunizing humans. Cholera toxin certainly would be another "useful" carrier, as would many of the other toxin molecules or subunits thereof that might be used to stimulate mucosal and/or parenteral immunity. Muller *et al.* (5) and Green and coworkers (6) studied the anti-influenza response after parenteral immunization of rabbits with conjugates of either hemocyanin or tetanus toxoid coupled to chemically synthesized peptides from the hemagglutinin molecule of influenza virus. In both cases, the antibodies raised to the peptide also bound to the intact virus particle. Lerner *et al.* looked at a similar system in rabbits (7) and in chimpanzees (8) in which they used a conjugate of hemocyanin and chemically synthesized peptides from the surface antigen of hepatitis B virus. Again, it was found that many of the anti-peptide antibodies cross-reacted with the intact viral envelope. In light of the above studies, and the fact that human exposure to many viral and bacterial pathogens first occurs at mucosal surfaces, it seems that it would be beneficial to produce protein conjugates consisting of viral and/or bacterial antigens coupled to an effective mucosal immunogen, such as CTB, for use as mucosal vaccines.

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